REMARKS

First, applicant appreciates the apparent withdrawal of rejections of certain claims under 35 U.S.C. § 102(e) as anticipated by Curtis (U.S. patent 5,824,780). There is essentially only one remaining rejection – that of claims 14-16 as assertedly obvious over Curtis as cited above in view of Livesey, *et al.* (U.S. patent 5,364,756). The rejection of all claims over these documents in combination with Bhattacharva, *et al.* (U.S. patent 5,288,853) will be argued on the same basis as the rejection of claims 14-16 over the combination of Curtis and Livesey. As noted in the previous response, the patentability of claims 20-22 is dependent on that of claims 14-16. Applicant believes that the rejection of claims 14-16 is in error for the following reasons.

No Motivation Has Been Demonstrated for Combination of Curtis with Livesey

The Examiner is entirely correct when he states that one cannot show non-obviousness by attacking references individually where the rejection is based on a combination of references. This rejection is based on such a combination, and as applicant is certain the Office would agree, motivation to combine the documents must be shown.

As outlined in *In re Rouffet*, 47 USPQ2d 1453 (Fed. Cir. 1998) (cited in the previous response), the Federal Circuit has delineated three acceptable rationales for making a combination. The first is that the suggestion to combine appears in the documents themselves. It does not appear that Livesey, which is focused on materials and methods for cryopreserving microscopic biological materials, suggests looking to Curtis, which is focused on converting native Factor VIII into active Factor VIII. Conversely, there appears to be no suggestion in Curtis that there is any need to examine other documents for especially effective methods of cryopreservation. Certainly, there is

no suggestion to consult a document which has to do with cryopreservation of cells and viruses. So the first rationale is not met.

The second rationale is that there is a common problem to be solved by each of the documents, especially if this common problem is the same as that attacked by the invention. This rationale is not met, either. Livesey is concerned with the problem of cryopreservation of cells and viruses; Curtis is directed to the problem of converting Factor VIII in its native form to Factor VIII in its activated form.

The third rationale is that at least one of the documents is so well-known in the art that it would automatically be on the mind of any practitioner. There is no assertion that either Curtis or Livesey is this type of document.

Applicant believes that the rejection is in error because no motivation for combination has been shown. A rationale must be provided for a rejection to be proper. *In re Rouffet (supra).* Applicant has reviewed the rejection set forth on pages 2-4, and does not find any articulation of any motivation to combine these documents. Similarly, applicant finds no response to his argument, set forth on page 10 of applicant's previous response, that none of the three *Rouffet* factors were present. For this reason alone, the rejection may properly be withdrawn.

Even If the Documents Are Combined, They Do Not Result In or Suggest the Invention as Claimed

Applicant is unable to discern in the statements in support of the rejection in what manner the combination of the features set forth in Curtis with those set forth in Livesey can be assembled to result in the invention as presently claimed. If the teachings of Curtis are combined with those of Livesey, the result would be that the activated form of Factor VIII, rather than native Factor VIII,

would be subjected to the cryopreservation method of Livesey. This is not what is claimed on a number of grounds.

First, as the Office agrees, the claims are limited to a method for preparing a stable dried composition of native Factor VIII, not activated Factor VIII. Therefore, the combination fails on that basis alone.

In addition, claim 14 requires a method of "freeze drying an aliquot of aqueous solution" of Factor VIII. The process disclosed in Livesey comprises freeze drying nebulized droplets of suspensions of Factor VIII. Applicant is a bit surprised that the Office fails to recognize the difference between a suspension and a solution, which applicant assumed would be well-known to any person of ordinary skill; however, if verification from a technical source is required, enclosed as Exhibit A are definitions of these terms from Steadman's Medical Dictionary and from a textbook on College Chemistry which (in case there was any doubt) verify that a solution is a homogeneous mixture where individual molecules of solutes cannot be observed and a suspension is a heterogeneous one where observable particles are, for at least a time, distributed in a medium. Thus, whatever justification there might be for subjecting Factor VIII or activated Factor VIII to the process of Livesey, the process of Livesey is not what is being claimed since an aliquot, not a nebulized droplet, and a solution, not a suspension, is freeze-dried.

It thus appears that not only does the combination of Livesey and Curtis fail to suggest the presence of trehalose in the absence of albumin, the combination fails even to suggest freeze drying of an aliquot of aqueous solution of native Factor VIII. The suggestion for just these limitations cannot arise from Livesey taken alone, and at best is argued to arise simply from the disclosure of

Curtis on the assumption that one of ordinary skill would find it obvious to substitute native Factor VIII for activated Factor VIII in whatever process might be disclosed in Curtis.

There Is No Specific Process for Freeze Drying Even Activated Factor VIII in Curtis

As a first observation, none of the examples set forth in Curtis involve freeze drying. Therefore, the statement made by the Office on page 6 of the Rejection that "Curtis establishes generally that Factor VIII has a therapeutic utility which can be preserved upon freeze drying in the presence of trehalose" is inaccurate on at least two grounds – no discussion of any stabilization of native Factor VIII appears anywhere in the document, and there is no actual demonstration that even activated Factor VIII can have its activity preserved upon freeze drying in the presence of trehalose.

As to the first deficiency, the Office states that one looking for suitable methods of preserving native Factor VIII would clearly consider Curtis' methods of preserving active Factor VIII relevant. This is contradicted by the enclosed declarations of Professor Ted Tuddenham and of Dr. Sam Helgerson, one of the inventors listed on the Curtis patent. Dr. Tuddenham outlines the dramatic differences between these proteins in paragraphs 3-5. As Professor Tuddenham states, there would be no expectation that such dissimilar proteins would behave in a similar way with regard to procedures for stabilization. Dr. Helgerson outlines in detail the differences between activated Factor VIII and native Factor VIII in paragraph 2 of his declaration.* And paragraph 3 states explicitly that the teaching of the Curtis patent was not intended to extend to native Factor VIII because any attempts at stabilization were aimed specifically at the particular arrangement of peptides in activated Factor VIII. As attested by both declarants, activated Factor VIII and native Factor VIII are very different proteins.

With regard to the second problem, while Curtis may imply that freeze drying may be used to preserve activated Factor VIII, no specific procedure, and certainly none that includes freeze-drying an aliquot of aqueous solution of this protein, is taught. Applicant finds only two references to freeze drying in the body of Curtis. One is in column 6, lines 34-37, which simply states that, "Lyophilization following activation, optionally followed by storage at a reduced temperature ... is effective in producing a stable activated Factor VIII preparation." The other is in column 5, lines 39-43, which simply says, "Following the preparation and stabilization of the activated Factor VIII, the protein can be lyophilized and stored at reduced temperatures until the protein is to be administered, at which time it can be redissolved in sterile solution for administration." The discussion in column 5 fails to suggest that stabilizers for lyophilization should include trehalose but not albumin. Both albumin and trehalose are listed as stabilizers. The paragraph even goes on to state that the specific activity of the compositions has to be calculated, taking into account any protein remaining in the composition.

The Office points to the claims in Curtis for the proposition that Curtis inherently discloses freeze drying the activated Factor VIII preparation in the presence of trehalose but the absence of albumin. Lyophilization is claimed in claim 5, which depends on claim 1. Claim 1 does not specify the addition of any stabilizer in the process of producing "activated and stabilized human Factor VIII protein." The steps in claim 1 are only an activating step, a step of removing the activating agent, other proteins and proteolytic fragments by adsorbing the activated Factor VIII onto a cationic exchange resin, maintaining a certain concentration of the activated Factor VIII and

* The document cited in paragraph 2 of Dr. Helgerson's declaration, Vehar, *et al., Nature* (1984) 312:337-342 is attached as Exhibit B.

eluting it from the resin using a buffer (not including histidine, incidentally) and adjusting the pH of the activated Factor VIII.

Thus claim 5, which depends simply from claim 1, does not specify including any stabilizing agent at all, trehalose or any other. In any event, if trehalose were included in the process of claim 1 during the activation step, it would be removed along with any albumin when the Factor VIII is adsorbed to the cation exchange resin, since trehalose is not a charged molecule. Even if claim 5 depended on claim 4, which requires the addition of a stabilizing additive during the activation process of claim 1, *i.e.*, before the step of adsorbing the activated Factor VIII to the cationic exchanger, the trehalose would not be present during the freeze drying step of claim 5.

Thus, even if one were to accept the unrealistic leap of faith that native Factor VIII would behave the same way as activated Factor VIII in terms of its ability to survive freeze drying under particular conditions, the method of the present invention is not suggested by Curtis, since Curtis makes no suggestion of the use of trehalose as a stabilizer in the absence of albumin at the time freeze drying occurs.

(It appears that this aspect of the Rejection as articulated by the Office is based on Curtis alone, not Curtis in combination with Livesey.)

Livesey Cannot Be Considered to Suggest the Present Invention

Again, the Office appears to base its conclusions entirely on the disclosure of Livesey taken alone, as it did in considering Curtis above. The arguments set forth beginning at page 3 of the Office action entirely concern Livesey and do not incorporate any teachings of Curtis. Livesey's process, even if applied to Factor VIII, as pointed out above, does not read on the present claims as it requires freeze drying nebulized droplets of a suspension of whatever is being cryopreserved, as

opposed to freeze drying an aliquot of an aqueous solution thereof. On its face, Livesey does not suggest the claimed process; Livesey teaches away.

The Office does not really address this point (except to sweep aside the different natures of solutions and suspensions), but argues that Livesey discloses that trehalose and not albumin is one of a number of agents particularly suited to dry preservation of macromolecules such as proteins, quoting column 9, lines 16-32. Applicant believes the very next paragraph should also have been quoted, which states that "Cryoprotectants, alone or in combination with other cryoprotectants or with additional components (for example, dry protectants) have also been found to be effective: ...." On this list is serum albumin plus trehalose! Thus, Livesey hardly appears to teach that proteins are preferably stabilized using trehalose in the absence of albumin.

Further support for the Office position is said to reside in Example 5, which putatively alone shows that trehalose, without albumin, is an effective cryoprotectant. First, Example 5 is so dramatically different from the process claimed that it is only marginally relevant. A soluble protein such as Factor VIII is not used in this process, but a virus which is clearly a particulate and appropriate to formation of a suspension. Second, an aliquot is not freeze dried — the "cryosolutions" are "rapidly frozen using the nebulizing device previously described." As has been pointed out previously, the cryosolutions are not themselves solutions, but rather a preparation which includes a suspension of biological material (see column 3, lines 53-56). Thus, neither the biological material to be preserved nor the process employed is a feature of the present claims.

Second, Example 5 fails to show any particular efficacy of trehalose in the absence of albumin as a cryoprotectant. As shown in the results of Example 5, set forth at the top of column 24, MES buffer alone is a more effective cryoprotectant than 250 mM trehalose for the virus

in the sample. Thus, buffer alone appears to be reasonably effective as well. Based on these results, one might conclude that trehalose has essentially no effect on stability upon freeze-drying.

It Is Not Permissible to Extract Only Portions of Documents Seen to be Related to the Invention and Recombine Them in a Manner Suggested Only by the Applicant

It appears that the Office has pieced together arbitrarily selected (from the standpoint of Curtis and Livesey) elements from materials and processes set forth in different contexts of the cited documents to construct a rejection based on the claimed invention. The Office may be arguing that since Example 5 of Livesey, which suggests a different process for a different biological material (not even a soluble protein) employs trehalose without albumin (although the trehalose appears not to be any more effective than buffer in preserving activity), this can legitimately be superimposed on the Factor VIII also mentioned in Livesey. The Office suggests that the use of trehalose alone, although in a different context, combined with native Factor VIII, although suggested in a different process, can be juxtaposed into the one or two vague allusions in Curtis to freeze drying an entirely different protein, activated Factor VIII, somehow to result in the claimed invention. Aside from the inadequacy of the combination itself, it appears that the selection of these elements is taught and motivated by the claimed subject matter. This is clearly improper. *W.L. Gore & Associates v. Garlock, Inc.*, 721 F2d 1540, 220 USPQ 303, 316 (Fed. Cir. 1983).

Summary

Respectfully, applicant requests reconsideration of the rejection of claims 14-16 over the combination of Curtis and Livesey. First, no rationale has been articulated for motivation to combine these documents. This alone is sufficient to demonstrate error. Second, when properly combined for what they actually teach, these documents do not suggest or teach the invention. The

documents taken alone or together do not even describe freeze drying an aliquot of aqueous solution of Factor VIII, much less the limitation that the solution contain trehalose and be free of albumin. The only illustrated use of trehalose taken alone is in a different process (nebulization of a suspension) of a different biological material (virus), and even in that context, trehalose does not appear to behave any better than buffer as a cryoprotectant. References to putative disclosures in either Curtis or Livesey suggesting that any freeze drying process involving Factor VIII (or even activated Factor VIII) should employ trehalose in the absence of albumin are so vague and uncertain that only the guidance of the invention would lead one to make this proposal. Accordingly, it is respectfully requested that this rejection be withdrawn, placing claims 14-22 in a position for allowance.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 559662000101.

Respectfully submitted,

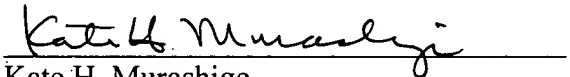
Dated: December 1, 2004 By: 

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EXHIBIT A

two volatile liquids is greater than that calculated from Raoult's law, the deviation is said to be positive, whereas when each vapor pressure is lower than the calculated value, the deviation is negative.

When deviation from Raoult's law is sufficiently extensive, there may be a range of concentrations in which the total vapor pressure of the solution is even higher than that of the more volatile component in the case of positive deviation, or even lower than that of the less volatile component in the case of negative deviation. In any such case there will be one particular solution that exhibits, respectively, a maximum or a minimum vapor pressure at a given temperature. The higher the vapor pressure of a particular solution at a given temperature, the lower will be its boiling point. Hence, when there are large positive deviations from Raoult's law, we may have a solution of minimum boiling point; with large negative deviations, we may have a solution of maximum boiling point.

The vapor from a solution of either minimum or maximum boiling point is of exactly the same composition as the solution itself. Hence, in these special cases, neither the composition nor the boiling point of the solution changes during distillation; on this account, such mixtures are called *constant boiling mixtures*. Alcohol and water, for example, form a minimum boiling mixture containing 95.5% of alcohol; nitric acid and water, a maximum boiling mixture containing 68% of nitric acid. Since constant boiling mixtures distill without change in composition, it is impossible in such cases to obtain both pure components from any solution by the process of fractional distillation.

THE COLLOIDAL STATE

15.22 Definitions

When a finely divided insoluble substance such as sand is shaken with water, a mixture is obtained that appears cloudy or turbid. On standing, however, the mixture soon becomes clear as the particles of sand settle to the bottom of the vessel. Such a mixture is called a *suspension*. On the other hand, if sugar or salt is stirred with water, the particles of solid completely disappear so that they cannot be observed even with the aid of the most powerful microscope; i.e., they go into solution.

Now, when powdered starch is treated with boiling water, or when arsenic(III) sulfide is formed by the passage of hydrogen sulfide through an aqueous solution of arsenic(III) oxide, a mixture is obtained that is not homogeneous, yet which shows scarcely any turbidity. The particles of insoluble solid (starch or arsenic(III) sulfide) do not settle out but remain in suspension indefinitely. Such a system, whose properties are intermediate between those of an ordinary suspension and those of a true solution, is called a *colloidal dispersion;* the finely divided substance is referred to as the *dispersed phase*, and the liquid is called the *dispersion medium*.

Although the term "colloid" (from the Greek word *kolla*—glue) was originally applied to a group of substances that readily form dispersions

Exhibit A

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26th Edition

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Exhibit A

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Cover Design: Sharon Reuter, Reuter & Associates

Database design by Lexi-Comp Inc., Hudson, OH
Printed in the United States of America by R.R. Donnelley & Sons Company

English Language Co-editions
Asian 1967, 1972, 1976
Indian 1967, 1973
Taiwan 1972, 1978

Translated Editions
Greek 1976
Indian 1977
Japanese 1977, 1985, 1995
Portuguese 1976, 1995
Spanish 1993

Library of Congress Cataloging-in-Publication Data

Stedman, Thomas Lathrop, 1853-1938.
[Medical dictionary]
Stedman's medical dictionary.—26th ed.
p. cm.
ISBN 0-683-07922-0 REGULAR EDITION
ISBN 0-683-07935-2 DELUXE EDITION
1. Medicine—Dictionaries. I. Title. II. Title: Medical dictionary.
[DNLM: 1. Dictionaries, Medical. W 13 S812m 1995]
R121.58 1995
610'.3—dc20
DNLM/DLC
for Library of Congress

97 98 99
3 4 5 6 7 8 9 10

Exhibit A

manipulation. **2.** The performance or procedures of an operation. [L. *chirurgia;* G. *cheir,* hand, + *ergon,* work]

ambulatory s., operative procedures performed on patients who are admitted to and discharged from a hospital on the same day.

aseptic s., the performance of an operation with sterilized hands, instruments, etc., and utilizing precautions against the introduction of infectious microorganisms from without.

closed s., s. without incision into skin, *e.g.,* reduction of a fracture or dislocation.

cosmetic s., s. in which the principal purpose is to improve the appearance, usually with the connotation that the improvement sought is beyond the normal appearance, and its acceptable variations, for the age and the ethnic origin of the patient. SYN esthetic s.

craniofacial s., simultaneous s. on the cranium and facial bones.

esthetic s., SYN cosmetic s.

featural s., rarely used term for plastic s. of the face, for correction or improvement of appearance.

keratorefractive s., SYN refractive *keratoplasty.*

laparoscopic s., operative procedure performed using minimally invasive surgical technique for exposure that avoids traditional incision. Visualization is achieved using a fiber optic instrument, usually attached to a video camera.

laparoscopically assisted s., operative procedure performed using combined laparoscopic and open techniques; most commonly applied to colon or small intestinal resections with anastomosis.

major s., SEE major *operation.*

microscopically controlled s., SYN Mohs' *chemosurgery.*

minimally invasive s., operative procedure performed in a manner derived to result in the smallest possible incision or no incision at all; includes laparoscopic, laparoscopically assisted, thoracoscopic, and endoscopic surgical procedures.

minor s., SEE minor *operation.*

Mohs' s., SYN Mohs' *chemosurgery.*

Mohs' micrographic s., SYN Mohs' *chemosurgery.*

open heart s., operative procedure(s) performed on or within the exposed heart, usually with cardiopulmonary bypass (as opposed to closed heart surgery).

oral s., the branch of dentistry concerned with the diagnosis and surgical and adjunctive treatment of diseases, injuries, and deformities of the oral and maxillofacial region.

orthognathic s., SYN surgical *orthodontics.*

orthopaedic s., the branch of s. that embraces the treatment of acute and chronic disorders of the musculoskeletal system, including injuries, diseases, dysfunction and deformities (orig. deformities in children) in the extremities and spine. SEE ALSO orthopaedics.

plastic s., the surgical specialty or procedure concerned with the restoration, construction, reconstruction, or improvement in the shape and appearance of body structures that are missing, defective, damaged, or misshapen.

reconstructive s., SEE plastic s.

stereotactic s., SYN stereotaxy.

thoracoscopic s., s. done using one or more endoscopic instruments.

transsexual s., procedures designed to alter a patient's external sexual characteristics so that they resemble those of the other sex.

video-assisted thoracic s. (VATS), a less morbid alternative to "open" thoracotomy that employs cameras, optic systems, percutaneous stapling devices, and assorted endoscopic graspers, retractors, and forceps. Also called video thoracoscopic surgery, it can be selectively applied to various pulmonary, pleural, and pericardial lesions.

sur·gi·cal (ser'ji-kăl). Relating to surgery.

sur·ra (ser'ă). A protozoan disease of camels, horses, mules, dogs, cattle, and other mammals in Africa, Asia, and Central and South America, caused by *Trypanosoma evansi;* infection is generally by mechanical transmission by a bloodsucking species of *Stomoxys* or *Tabanus.* SEE ALSO murrina. [East Indian name]

sur·re·nal (ser-rē'năl). SYN suprarenal (1).

sur·ro·gate (ser'ŏ-gāt). **1.** A person who functions in another's life as a substitute for some third person such as a relative w assumes the nurturing and other responsibilities of the abs parent. **2.** A person who reminds one of another person so t one uses the first as an emotional substitute for the second. [*surrogo,* to put in another's place]

mother s., one who substitutes for or takes the place of t mother.

sur·sa·nure (ser-sā'nūr). A superficially healed ulcer, with p beneath the surface. [Fr., fr. L. *super,* over, + *sanus,* healthy]

sur·sum·duc·tion (ser-sŭm-dŭk'shŭn). SYN supraduction. [*sursum,* upward, + *duco,* pp. *-ductus,* to draw]

sur·sum·ver·sion (ser-sŭm-ver'zhŭn). The act of rotating t eyes upward. [L. *sursum,* upward, + *verto,* pp. *versus,* to turn]

sur·veil·lance (ser-vā'lans). **1.** The collection, collation, analy sis, and dissemination of data; a type of observational study th involves continuous monitoring of disease occurrence within population. **2.** Ongoing scrutiny, generally using methods distin guished by practicability, uniformity, rapidity, rather than com plete accuracy. [Fr. *surveiller,* to watch over, fr. L. *super- vigilo,* to watch]

> Surveillance does not aim for accuracy or completeness; rather it is designed to provide practical and uniform results in a timely fashion, so that trends can be spotted and appropriate action taken. Such action might include further investigation of some aspect of an unfolding phenomenon, or even intervention. Surveillance is employed frequently in the monitoring of disease or factors influencing disease. The data being analyzed and interpreted may include 1) mortality and morbidity reports based on death certificates, hospital records, or general practice sentinels or notifications; 2) laboratory test results; 3) disease outbreak reports; 4) vaccine utilization-uptake and side effects; 5) reports of work- or school-related absences due to illness; (6) biological changes in known agents, vectors, or reservoirs of disease.

immune s., A theory that the immune system destroys tumor cells which are constantly arising during the life of the individual. SYN immunological s.

immunological s., SYN immune s.

post-marketing s., procedure implemented after a drug has been licensed for public use, designed to provide information on use and on occurrence of side effects, adverse effects, etc.

sur·vey (ser'vā). **1.** An investigation in which information is systematically collected but in which the experimental method is not used. **2.** a comprehensive examination or group of examinations to screen for one or more findings. **3.** a series of questions administered to a sample of individuals in a population. [O.Fr. *surveeir,* fr. Mediev.L. *supervideo,* fr. *super,* over, + *video,* to see]

field s., the planned collection of data among noninstitutionalized persons in the general population.

skeletal s., radiographic examination of all or selected parts of the skeleton, as for occult fractures, metastases, etc.

sur·vey·ing (ser-vā'ing). In dentistry, the procedure of locating and delineating the contour and position of the abutment teeth and associated structures before designing a removable partial denture.

sur·vey·or (ser-vā'er, ōr). In dentistry, the instrument used in surveying.

sur·viv·al (ser-vī'văl). Continued existence; persistence of life.

sus·cep·ti·bil·i·ty (su-sep-ti-bil'i-tē). **1.** Likelihood of an individual to develop ill effects from an external agent, such as *Mycobacterium tuberculosis,* high altitude, or ambient temperature. **2.** In magnetic resonance imaging, the loss of magnetization signal caused by rapid phase dispersion because of marked local inhomogeneity of the magnetic field, as with the multiple air-soft tissue interfaces in the lung; s. measurement can estimate calcium content in trabecular bone.

sus·pen·sion (sŭs-pen'shŭn). **1.** A temporary interruption of any function. **2.** A hanging from a support, as used in the treatment of spinal curvatures or during the application of a plaster jacket. **3.**

Exhibit A

Fixation of an organ, such as the uterus, to other tissue for support. **4.** The dispersion through a liquid of a solid in finely divided particles of a size large enough to be detected by purely optical means; if the particles are too small to be seen by microscope but still large enough to scatter light (Tyndall phenomenon), they will remain dispersed indefinitely and are then called a colloidal s. SYN coarse dispersion. **5.** A class of pharmacopeial preparations of finely divided, undissolved drugs (*e.g.*, powders for s.) dispersed in liquid vehicles for oral or parenteral use. [L. *suspensio*, fr. *sus-pendo*, pp. *-pensus*, to hang up, suspend]

amorphous insulin zinc s., SYN prompt insulin zinc s.

chromic phosphate P 32 colloidal s., a pure β-emitting colloidal, nonabsorbable radiopharmaceutical administered into body cavities such as the pleural or peritoneal spaces to control malignant effusions. SEE ALSO *sodium* phosphate P 32.

Coffey s., an operative technique following partial excision of the cornu, as in salpingectomy, whereby the broad and the round ligament are sutured over the cornual wound to restore continuity of the peritoneum and to suspend the uterus on the operated side.

crystalline insulin zinc s., SYN extended insulin zinc s.

extended insulin zinc s., a long-acting insulin s., obtained from beef, with an approximate time of onset of 7 hours and a duration of action of 36 hours. SYN crystalline insulin zinc s.

insulin zinc s., a sterile buffered s. with zinc chloride, containing 40 or 80 units per ml; the solid phase of the s. consists of a mixture of 7 parts of crystalline insulin and 3 parts of amorphous insulin. SYN lente insulin.

magnesia and alumina oral s., a mixture of magnesium hydroxide and variable amounts of aluminum oxide; used as an antacid.

prompt insulin zinc s., sterile s. of insulin in buffered water for injection, modified by the addition of zinc chloride such that the solid phase of the s. is amorphous; it contains 40 or 80 units per ml; the duration of action is equivalent to that of insulin injection. SYN amorphous insulin zinc s., semilente insulin.

sus·pen·soid (sŭs-pen'soyd). A colloidal solution in which the disperse particles are solid and lyophobe or hydrophobe, and are therefore sharply demarcated from the fluid in which they are suspended. SYN hydrophobic colloid, lyophobic colloid, suspension colloid. [suspension + G. *eidos*, resemblance]

sus·pen·so·ry (sŭs-pen'sŏ-rē). **1.** Suspending; supporting; denoting a ligament, a muscle, or other structure that keeps an organ or other part in place. **2.** A supporter applied to uplift a dependent part, such as the scrotum or a pendulous breast.

sus·ten·tac·u·lar (sŭs-ten-tak'yū-lăr). Relating to a sustentaculum; supporting.

sus·ten·tac·u·lum, pl. **sus·ten·tac·u·la** (sŭs'ten-tak'yū-lŭm, -lă) [NA]. A structure that serves as a stay or support to another. [L. a prop, fr. *sustento*, to hold upright]

s. li'enis, SYN splenorenal *ligament*.

s. ta'li [NA], support of the talus, a bracket-like lateral projection from the medial surface of the calcaneus, the upper surface of which presents a facet for articulation with the talus.

su·sur·rus (sŭ-ser'ŭs). SYN murmur (1). [L.]

s. au'rium, murmur in the ear.

Sutter blood group. See Blood Groups appendix.

Sutton, Richard L., U.S. dermatologist, 1878–1952. SEE S.'s *disease* (1), *nevus*.

Sutton, Richard L., Jr., U.S. dermatologist, *1908. SEE S.'s *disease* (2), *ulcer*.

SUTURA

su·tu·ra, pl. **su·tu·rae** (sū'tū'ră, -rē) [NA]. SYN suture. [L. a sewing, a suture, fr. *suo*, pp. *sutus*, to sew]

s. corona'lis [NA], SYN coronal *suture*.

sutu'rae cra'nii [NA], SYN cranial *sutures*, under *suture*.

s. ethmoidolacrima'lis [NA], SYN ethmoidolacrimal *suture*.

s. ethmoidomaxilla'ris [NA], SYN ethmoidomaxillary *suture*.

s. fronta'lis [NA], SYN frontal *suture*.

s. frontoethmoida'lis [NA], SYN frontoethmoidal *suture*.

s. frontolacrima'lis [NA], SYN frontolacrimal *suture*.

s. frontomaxilla'ris [NA], SYN frontomaxillary *suture*.

s. frontonasa'lis [NA], SYN frontonasal *suture*.

s. frontozygomat'ica [NA], SYN frontozygomatic *suture*.

s. inci'siva [NA], SYN incisive *suture*.

s. infraorbita'lis, SYN infraorbital *suture*.

s. intermaxilla'ris [NA], SYN intermaxillary *suture*.

s. internasa'lis [NA], SYN internasal *suture*.

s. interparieta'lis, SYN sagittal *suture*.

s. lacrimoconcha'lis [NA], SYN lacrimoconchal *suture*.

s. lacrimomaxilla'ris [NA], SYN lacrimomaxillary *suture*.

s. lambdoi'dea [NA], SYN lambdoid *suture*.

s. meto'pica [NA], SYN metopic *suture*.

s. nasofronta'lis, SYN frontonasal *suture*.

s. nasomaxilla'ris [NA], SYN nasomaxillary *suture*.

s. no'tha (nō'tă), SYN false *suture*. [G. fem. of *nothos*, spurious]

s. occipitomastoi'dea [NA], SYN occipitomastoid *suture*.

s. palati'na media'na [NA], SYN median palatine *suture*.

s. palati'na transver'sa [NA], SYN transverse palatine *suture*.

s. palatoethmoida'lis [NA], SYN palatoethmoidal *suture*.

s. palatomaxilla'ris [NA], SYN palatomaxillary *suture*.

s. parietomastoi'dea [NA], SYN parietomastoid *suture*.

s. pla'na [NA], SYN plane *suture*.

s. sagitta'lis [NA], SYN sagittal *suture*.

s. serra'ta [NA], SYN serrate *suture*.

s. sphenoethmoida'lis [NA], SYN sphenoethmoidal *suture*.

s. sphenofronta'lis [NA], SYN sphenofrontal *suture*.

s. sphenomaxilla'ris [NA], SYN sphenomaxillary *suture*.

s. spheno-orbita'lis, SYN spheno-orbital *suture*.

s. sphenoparieta'lis [NA], SYN sphenoparietal *suture*.

s. sphenosquamo'sa [NA], SYN sphenosquamous *suture*.

s. sphenovomeria'na [NA], SYN sphenovomerine *suture*.

s. sphenozygoma'tica [NA], SYN sphenozygomatic *suture*.

s. squamo'sa [NA], **(1)** SYN squamous *suture*. **(2)** SYN squamoparietal *suture*.

s. squamosomastoi'dea [NA], SYN squamomastoid *suture*.

s. temporozygomat'ica [NA], SYN zygomaticotemporal *suture*.

s. zygomaticofronta'lis, SYN frontozygomatic *suture*.

s. zygomaticomaxilla'ris [NA], SYN zygomaticomaxillary *suture*.

s. zygomaticotempora'lis, SYN zygomaticotemporal *suture*.

su·tur·al (sū'chūr-ăl). Relating to a suture in any sense.

SUTURE

su·ture (sū'chūr). **1.** A form of fibrous joint in which two bones formed in membrane are united by a fibrous membrane continuous with the periosteum. **2.** To unite two surfaces by sewing. SYN stitch (3). **3.** The material (silk thread, wire, catgut, etc.) with which two surfaces are kept in apposition. **4.** The seam so formed, a surgical s. SYN sutura [NA], suture joint. [L. *sutura*, a seam]

absorbable surgical s., a surgical s. material prepared from a substance that can be digested by body tissues and is therefore not permanent; it is available in various diameters and tensile strengths, and can be treated to modify its resistance to absorption and be impregnated with antimicrobial agents.

Albert's s., a modified Czerny s., the first row of stitches passing through the entire thickness of the wall of the gut.

apposition s., a s. of the skin only. SYN coaptation s.

approximation s., a s. that pulls together the deep tissues.

atraumatic s., a s. swaged onto the end of an eyeless needle.

Exhibit A

and his followers that disease was due to an imbalance between solid particles (atoms) of the body and the spaces (pores) between them, a doctrine which opposed the humoral conception of Hippocrates. SYN methodism.

sol·id·ist (sol'i-dist). An adherent of the doctrine of solidism.

sol·id·is·tic (sol-i-dis'tik). Relating to solidism.

sol·i·dus (sol'i-dŭs). That line on a constitution diagram indicating the temperature below which all metal is solid.

sol·i·ped (sol'i-ped). A solid-hoofed animal such as the horse. [L. *solidus*, solid, + *pes*, foot]

sol·ip·sism (sō'lip-sizm, sol'ip-). A philosophical concept that whatever exists is a product of will and the ideas of the perceiving individual. [L. *solus*, alone, + *ipse*, self]

soln. Abbreviation for solution.

sol·u·bil·i·ty (sol-yū-bil'i-tē). The property of being soluble.

sol·u·ble (sol'yū-bl). Capable of being dissolved. [L. *solubilis*, fr. *solvo*, to dissolve]

so·lum (sō'lŭm). Bottom; the lowest part. [L.]

sol·ute (sol'yūt, sō'lūt). The dissolved substance in a solution. [L. *solutus*, dissolved, pp. of *solvo*, to dissolve]

so·lu·tio (sō-lū'shē-ō). SYN solution. [L.]

so·lu·tion (sol., soln.) (sō-lū'shŭn). **1.** The incorporation of a solid, a liquid, or a gas in a liquid or noncrystalline solid resulting in a homogeneous single phase. SEE dispersion, suspension. **2.** Generally, an aqueous s. of a nonvolatile substance. **3.** In the language of the Pharmacopeia, an aqueous s. of a nonvolatile substance is called a solution or liquor; an aqueous s. of a volatile substance is a water (aqua); an alcoholic s. of a nonvolatile substance is a tincture (tinctura); an alcoholic s. of a volatile substance is a spirit (spiritus); a s. in vinegar is a vinegar (acetum); a s. in glycerin is a glycerol (glyceritum); a s. in wine is a wine (vinum); a s. of sugar in water is a syrup (syrupus); a s. of a mucilaginous substance is a mucilage (mucilago); a s. of an alkaloid or metallic oxide in oleic acid is an oleate (oleatum). **4.** The termination of a disease by crisis. **5.** A break, cut, or laceration of the solid tissues. SEE s. of contiguity, s. of continuity. SYN solutio. [L. *solutio*]

acetic s., a vinegar.

amaranth s., a 1% s. of amaranth (trisodium naphthol sulfonic acid), a synthetic vivid red dye, stable in acid and intensified in sodium hydroxide s.; used as a red or pink colorant in liquid pharmaceuticals.

aqueous s., a s. containing water as the solvent; examples include lime water, rose water, saline s., and a large number of s.'s intended for intravenous administration.

Benedict's s., an aqueous solution of sodium citrate, sodium carbonate, and copper sulfate which changes from its normal blue color to orange, red, or yellow in the presence of a reducing sugar such as glucose. SEE ALSO Benedict's *test* for glucose.

Burow's s., a preparation of aluminium subacetate and glacial acetic acid, used for its antiseptic and astringent action on the skin.

chemical s., SEE solution (1).

colloidal s., a dispersoid, emulsoid, or suspensoid. SYN colloidal dispersion.

s. of contiguity, the breaking of contiguity; a dislocation or displacement of two normally contiguous parts.

s. of continuity, division of bones or soft parts that are normally continuous, as by a fracture, a laceration, or an incision. SYN diastasis.

Dakin's s., a bactericidal wound irrigant. SYN Dakin's fluid.

disclosing s., a s. that selectively stains all soft debris, pellicle, and bacterial plaque on teeth; used as an aid in identifying bacterial plaque after rinsing with water.

Earle's s., a tissue culture medium containing $CaCl_2$, $MgSO_4$, $NaHCO_3$, NaCl, $NaH_2PO_4 \cdot H_2O$, and glucose.

ethereal s., a s. of any substance in ether.

Fehling's s., an alkaline copper tartrate s. formerly used for detection of reducing sugars. SYN Fehling's reagent.

ferric and ammonium acetate s., a clear, aromatic, reddish-brown liquid which has been used in iron-deficiency anemia in children and man; a source of iron. SYN Basham's mixture.

Fonio's s., a diluent with magnesium sulfate, used for stained smears of blood platelets.

Gallego's differentiating s., a dilute s. of formaldehyde and acetic acid used in a modified Gram stain to differentiate and enhance the basic fuchsin binding to Gram-negative microorganisms.

Gey's s., a salt s. usually used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells.

Hanks' s., a salt s. usually used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells; two variations contain $CaCl_2$, $MgSO_4 \cdot 7H_2O$, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $Na_2HPO_4 \cdot 2H_2O$, and D-glucose.

Hartmann's s., SYN lactated Ringer's s.

Hartman's s., a s. used to desensitize dentin in dental operations; contains thymol, ethyl alcohol, and sulfuric ether.

Hayem's s., a blood diluent used prior to counting red blood cells.

Krebs-Ringer s., a modification of Ringer's s., prepared by mixing NaCl, KCl, $CaCl_2$, $MgSO_4$, and phosphate buffer, pH 7.4.

lactated Ringer's s., a s. containing NaCl, sodium lactate, $CaCl_2$(dihydrate), and KCl in distilled water; used for the same purposes as Ringer's s. SYN Hartmann's s.

Lange's s., a colloidal gold s. used to demonstrate protein abnormalities in spinal fluid. SEE Lange's *test*.

Locke-Ringer s., a s. containing NaCl, $CaCl_2$, KCl, $MgCl_2$, $NaHCO_3$, D-glucose, and water; used in the laboratory for physiological and pharmacological experiments.

Locke's s.'s, s.'s containing, in varying amounts, NaCl, $CaCl_2$, KCl, $NaHCO_3$, and D-glucose; used for irrigating mammalian heart and other tissues, in laboratory experiments; also used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells.

Lugol's iodine s., an iodine-potassium iodide s. used as an oxidizing agent, for removal of mercurial fixation artifacts, and also in histochemistry and to stain amebas.

molecular dispersed s., SYN dispersoid.

Monsel s., ferric subsulfate s. used to coagulate superficial bleeding such as that following skin biopsy.

normal s., SEE normal (3).

ophthalmic s.'s, sterile s.'s, free from foreign particles and suitably compounded and dispensed for instillation into the eye.

Ringer's s., **(1)** a s. resembling the blood serum in its salt constituents; it contains 8.6 g of NaCl, 0.3 g of KCl, and 0.33 g of $CaCl_2$ in each 1000 ml of distilled water; used topically for burns and wounds; **(2)** a salt s. usually used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells. SEE Ringer's *injection*.

saline s., **(1)** a s. of any salt; SYN salt s. **(2)** specifically, an isotonic sodium chloride s.; 0.85 to 0.9/100 ml water.

salt s., SYN saline s. (1).

saturated s. (sat. sol., sat. soln.), a s. that contains all of a substance capable of dissolving; a solution of a substance in equilibrium with an excess undissolved substance.

standard s., standardized s., a s. of known concentration, used as a standard of comparison or analysis.

supersaturated s., a s. containing more of the solid than the liquid would ordinarily dissolve; it is made by heating the solvent when the substance is added, and on cooling the latter is retained without precipitation; addition of a crystal or solid of any kind usually results in precipitation of the excess solute, leaving a saturated s.

test s., a s. of some reagent, in definite strength, used in chemical analysis or testing.

Tyrode's s., a modified Locke's s.; it contains 8 g of NaCl, 0.2 g of KCl, 0.2 g of $CaCl_2$, 0.1 g of $MgCl_2$, 0.05 g of NaH_2PO_4, 1 g

Exhibit A

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Structure of human factor VIII

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The deduced amino acid sequence of human factor VIII, obtained from the DNA sequence, predicts a mature polypeptide of 2,332 amino acids containing a triplicated domain structure. The polypeptide has 35% sequence homology with the copper-binding plasma protein, ceruloplasmin. Determination of the thrombin cleavage sites in plasma-derived factor VIII polypeptides allows prediction of the domains involved in the associated activation and inactivation of the protein.

PREPARATIONS of factor VIII/von Willebrand factor complex[1-12] contain four closely related properties or activities: factor VIII coagulant activity, an antigen associated with the factor VIII coagulant activity, platelet adhesion promoting activity and an antigen precipitated by antisera raised against the purified complex (factor VIII-related protein). Factor VIII separated from the complex has associated trace amounts of protein, is unstable and consists of multiple polypeptide chains[13-17], hindering detailed characterization studies.

The purification of human factor VIII by affinity to a monoclonal antibody directed against the coagulant activity of factor VIII[18], has allowed characterization of the protein fragments of factor VIII or thrombin-activated factor VIII by partial amino acid sequence analysis. This sequence information has been used to isolate cDNA and genomic clones encoding human factor VIII[19,20]. The protein sequence deduced from these clones, together with an analysis of the cleavage products associated with the activation of factor VIII by thrombin, allows the assignment of thrombin cleavage sites and the identification of most of the polypeptide fragments present in highly purified factor VIII preparations. The factor VIII sequence exhibits striking homology with the plasma copper-binding protein ceruloplasmin, suggesting novel biochemical activities for factor VIII as well as a role for metal ions other than calcium in the blood coagulation cascade.

Analysis of plasma factor VIII

Preparations of human factor VIII[18] purified over 300,000-fold from plasma contained several proteins of relative molecular mass (M_r) 210,000–80,000 (Fig. 1*A*). These protein bands were not connected by disulphide links because samples analysed under non-reducing conditions gave a similar pattern (data not shown). To determine the relationship of these multiple polypeptide chains, we analysed them by tryptic peptide mapping. The preparation used in Fig. 1*B* contained a protein of M_r 240,000, producing a peptide map which did not show identity with the other proteins of the mixture (Fig. 1*B*, *a*), and has been found to be a von Willebrand factor subunit (data not shown). Peptides of M_r 90,000–210,000 all had a common tryptic map, indicating that they are derived from the same or closely related polypeptide chains (Fig. 1*B*, *b-f*). Furthermore, Western blot analysis of the factor VIII preparations demonstrated that a factor VIII-specific monoclonal antibody[18] reacted with the M_r 90,000–210,000 polypeptides (data not shown). Two very similar patterns generated by the proteins of M_r 80,000 and 70,000 had a different peptide map (Fig. 1*B*, *g*, *h*). These results demonstrate that the fragments of M_r 90,000–210,000 are structurally related and could be pooled and treated as one polypeptide chain. The protein represented by the band of M_r 80,000 (and 70,000 when present) was analysed separately.

The purified factor VIII preparations were fractionated by gel filtation on a TSK 4000SW HPLC column; analysis of the resulting fractions demonstrated the effective separation of M_r 240,000 protein, the polypeptides of M_r 90,000–210,000 and the M_r 80,000 fragment (Fig. 2*B*). We performed amino acid sequence analysis on peptides generated from the M_r 80,000 protein, the polypeptide pool of M_r 90,000–210,000 and a fragment of M_r 90,000 from limited thrombin digestion of the M_r 90,000–210,000 pool. After digestion of each sample with trypsin, the resulting peptides were separated by reverse-phase HPLC and sequenced. The peptide sequence AWAYFSDVDLEK, used to prepare synthetic DNA probes identifying factor VIII genomic DNA clones, is indicated in Fig. 2*C*.

Structure of factor VIII protein

The molecular cloning of the entire factor VIII coding region is described in an accompanying paper[19]. The 2,351-amino acid sequence for factor VIII, deduced from the nucleotide sequence of these clones, is shown in Fig. 3. The first 19 amino acids of the sequence comprise the signal sequence for factor VIII, based on peptide sequence analysis of a fragment derived from the M_r 90,000–210,000 polypeptide pool. The N-terminal sequence of this M_r 30,000 fragment, obtained as a thrombin digest product of the M_r 90,000–210,000 pool, is identical to the first 12 amino acids which follow the predicted factor VIII leader sequence (see Fig. 5). This presequence exhibits a core of 10 hydrophobic amino acids flanked by two charged residues, a structure which conforms to that observed for the leader sequences found in most secreted proteins[21]. The mature protein contains 2,332 amino acids (calculated M_r 264,763).

The availability of the complete factor VIII sequence reveals the organization and identity of the tryptic peptides obtained from the pools of separated plasma-derived factor VIII fragments. Essentially all tryptic peptide sequences determined from the M_r 90,000–210,000 protein pools are located in the amino-

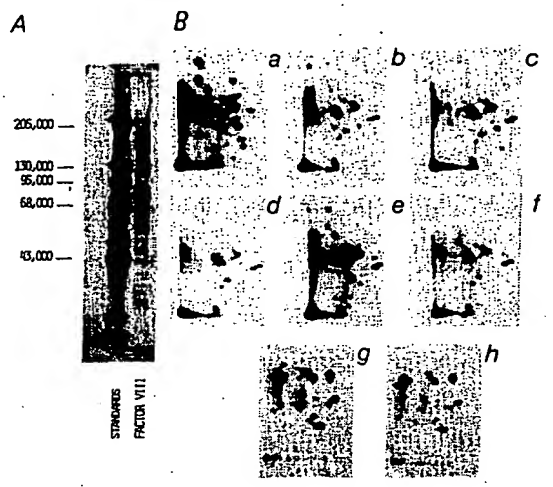

Fig. 1 *A*, Purified human factor VIII analysed by SDS-polyacrylamide gel electrophoresis. *B*, Two-dimensional tryptic mapping of factor VIII polypeptide chains. The resulting tryptic patterns of proteins of M_r: *a*, 240,000; *b*, 210,000; *c*, 170,000; *d*, 150,000; *e*, 120,000; *f*, 100,000; *g*, 80,000; and *h*, 70,000 are shown.
Methods: The purified protein[18] was reduced and analysed in a 5–10% polyacrylamide gradient gel in the presence of SDS by the procedure of Laemmli[37]. The molecular weights of the protein standards are shown (myosin, β-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin). The bands were detected using the silver stain procedure of Morrissey[38]. 1 μg of a factor VIII preparation was denatured in 1% SDS and labelled with 300 μCi ^{125}I for 15 min using the iodobead procedure[38]. Labelled polypeptides were located on dried SDS-polyacrylamide gels by autoradiography and digested by incubation of gel slices with 10 μg trypsin in 0.1 M ammonium bicarbonate buffer for 6 h at 37°C. After repeated lyophilizations, peptides were dissolved in 8.8% formic acid and a portion was subjected to thin-layer electrophoresis in the same buffer (400 V for 45 min) on pre-coated TLC-cellulose platers (E. Merck, Darmstadt, FRG). For the second dimension, peptides were separated by ascending chromatography in *n*-butanol/pyridine/glacial acetic acid/water, 75:50:15:60 (v/v). The plates were then subjected to autoradiography.

terminal half of the molecule, whereas those sequences obtained for the M_r 80,000 fragment are found at the carboxy-terminus of the factor VIII sequence (unpublished results). The most carboxy-terminal tryptic peptides identified for the M_r 90,000–210,000 pool gave the sequences GEFT and −QEE, beginning at positions 1,155 and 1,194, respectively. This shows that the M_r 210,000 fragment consists of a protein of $M_r \geq 135{,}000$ containing 14 potential asparagine-linked glycosylation sites. The location of the M_r 80,000 fragment of factor VIII is delineated by two peptide sequences which define a stretch of ~680 amino acids. The first of these was obtained from the amino-terminal sequence of the M_r 80,000 fragment beginning at position 1,649 (see Fig. 5), whereas the second corresponds to a tryptic peptide, MEVLGCEAQDL, 12 amino acids from the C-terminus predicted by the DNA sequence. Thus, there is no significant removal of C-terminal sequences from the plasma-derived molecule. The failure to recover tryptic peptide sequences from the region between position 1,200 and the M_r 80,000 fragment is probably due to the relatively low concentration of the M_r 210,000 species in the M_r 90,000–210,000 fragment pool. This position should therefore be considered the minimal C-terminal extent of the M_r 210,000 protein.

Computer-aided analysis of the factor VIII protein sequence revealed two types of internal homology: the first consists of a triplicated segment (A domain) found at positions 1–329, 380–711 and 1,649–2,019 of the mature polypeptide (Fig. 4); the second and third domains of the triplication are separated by a region of 983 amino acids (B domain) extremely rich in potential asparagine-linked glycosylation sites. In addition, an unrelated duplication of 150 amino acids is found at the C-terminus of the molecule (C domain). The A domains have ~30% amino acid homology, whereas the C domains are ~40% homologous. Most

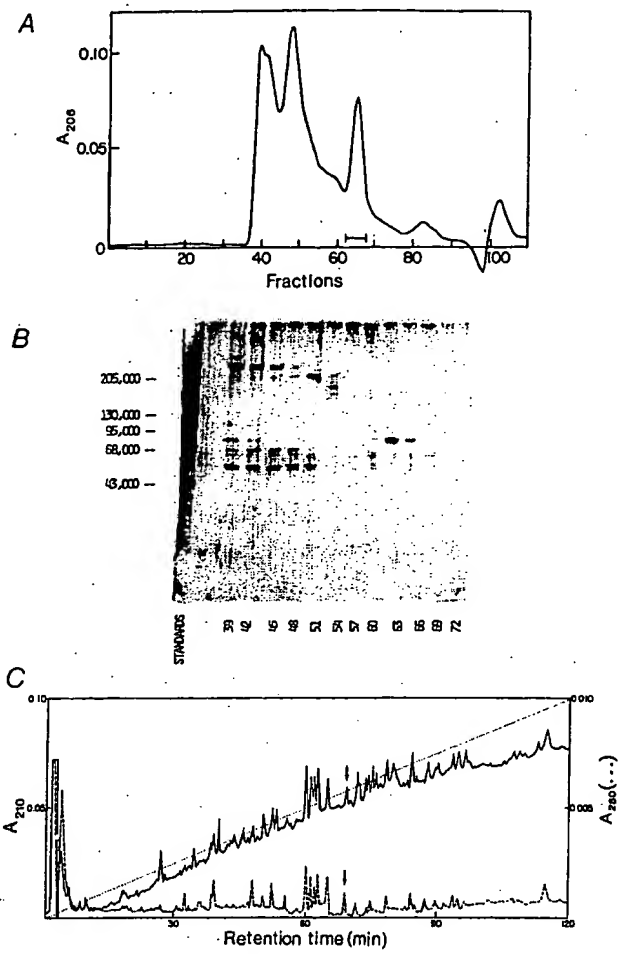

Fig. 2 Purification of factor VIII polypeptides. *A*, Fractionation of proteins by TSK 4000 HPLC. *B*, SDS-polyacrylamide gel analysis of fractions from TSK 4000 chromatography. *C*, Reverse-phase HPLC separation of tryptic peptides of M_r 80,000 protein.
Methods: *A*, Human factor VIII preparations[18] were dialysed into 1% ammonium bicarbonate containing 0.1% SDS. The samples were lyophilized and stored at −20°C until use. The samples were reconstituted in distilled water and applied to a TSK 4000 SW column (0.75 × 50 cm; Alltech Associates, Deerfield, Illinois) equilibrated with 0.1 M sodium phosphate buffer, *p*H 7.0, containing 0.1% SDS. Samples of ~0.15–0.25 ml were injected and the column developed isocratically at a flow rate of 0.5 ml min^{-1}. Absorbance was monitored at either 206 nm or 280 nm and fractions (0.2 ml) collected. Fractions across the profile were reduced and analysed by SDS-polyacrylamide gel electrophoresis as described in Fig. 1 legend. The protein of M_r 80,000 was pooled for tryptic digestion as shown in *A*. *C*, The TSK 4000 SW purified protein of M_r 80,000 (0.8 nM) was dialysed under a nitrogen atmosphere overnight against 0.36 M Tris-HCl, *p*H 8.6, containing 8 M urea, 3.3 mM EDTA and 10 mM dithiothreitol, DTT (final vol. 1.5 ml). The protein was alkylated by adding 15 μl of 5 M iodoacetic acid (dissolved in 1 M NaOH). The reaction was allowed to proceed for 35 min at room temperature in the dark and was quenched by adding DTT to a final concentration of 100 mM. The protein solution was dialysed against 8 M urea in 0.1 M ammonium bicarbonate for 4 h. The urea dialysis solution was changed over a period of 24 h to gradually reduce the urea concentration to a final level of 0.5 M. Trypsin was added at a weight ratio of 1:50 at 37°C for 12 h. HPLC separation of the resulting tryptic peptides was performed on a high-resolution Synchropak RP-P C-18 column (0.46 × 25 cm; 10 μ). The column was developed with a gradient of acetonitrile (1–70% in 200 min) in 0.1% trifluoroacetic acid. Absorbance was monitored at 210 nm and 280 nm. Each peak was collected and stored at 4°C until subjected to sequence analysis in a Beckman spinning cup sequencer with on-line phenylthiohydantoin amino acid identification[40]. The arrow identifies the peptide (AWAYFSDVDLEK) resulting in identification of a factor VIII genomic clone[19,20].

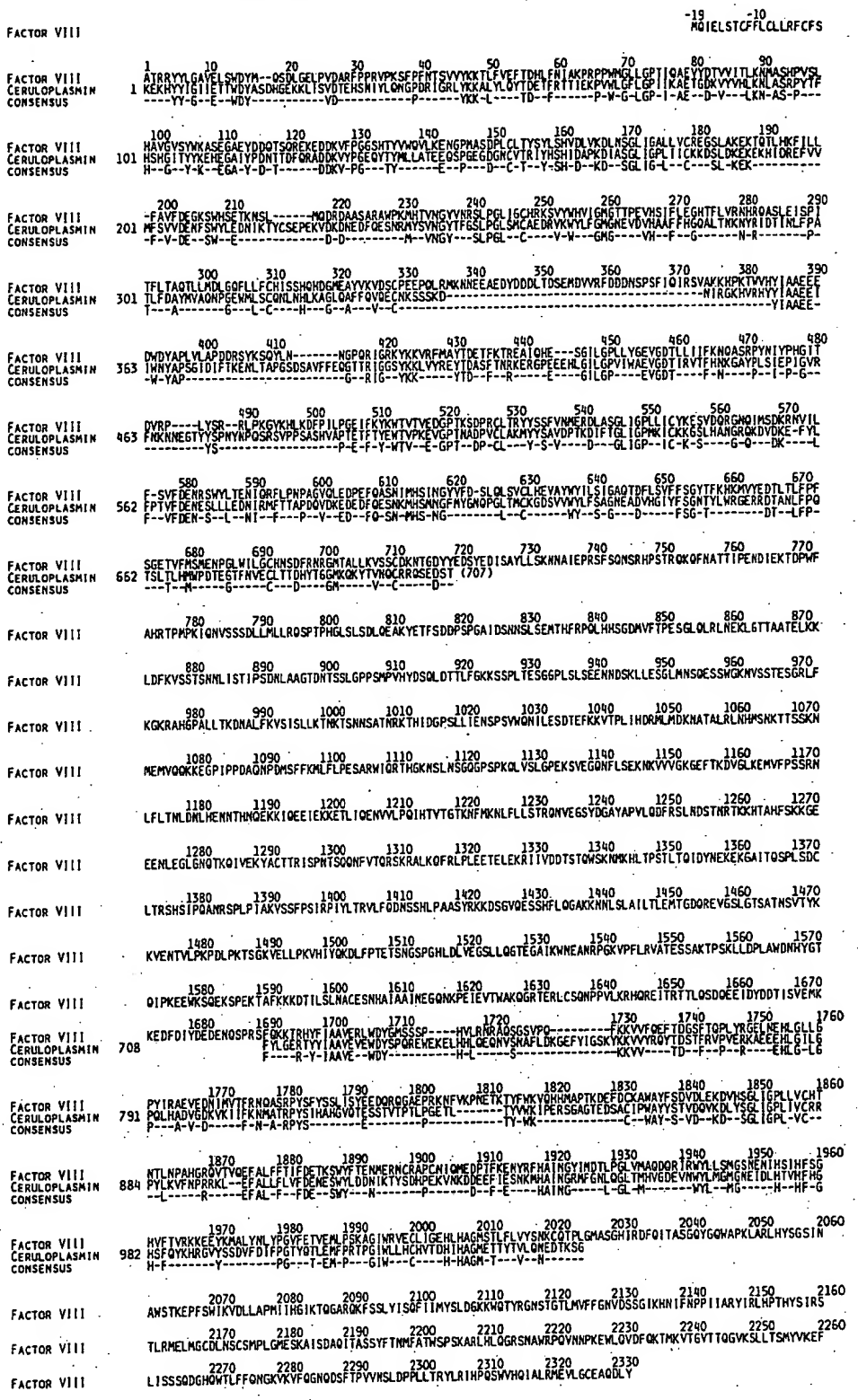

Fig. 3 Homology of human factor VIII with human ceruloplasmin. The deduced factor VIII amino acid sequence derived from cDNA and genomic clones[19,20] is compared with that of human ceruloplasmin[22]. The consensus line shows residues which are identical in the two proteins. The numbering above the sequences is that of factor VIII. The number preceding the ceruloplasmin line represents the numbering of the amino acid sequence beginning the line. The single-letter notation for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

of the 23 cysteine residues of the mature polypeptide are clustered in the A and C domains and occupy similar positions Fig. 6), suggesting that the structures of both repeated domains of factor VIII reflect conserved disulphide bonding arrangements.

The A domains of the factor VIII protein show striking homology with the copper-binding plasma protein ceruloplasmin (Fig. 3). Amino acid sequence analysis of ceruloplasmin has revealed a structure consisting of three contiguous domains sharing ~30% homology[22-24]. The triplicated domains of factor VIII and ceruloplasmin exhibit a pairwise homology of 30% (Fig. 4). Although the B domain has no substantial homology with any known sequence, the C domain shares 20% amino acid homology with the discoidin lectins from *Dictyostelium*[25].

Ceruloplasmin contains six copper atoms in three distinct types of coordination: two of type 1, one of type 2 and three electron paramagnetic resonance-nondetectable type 3 copper ions[26]. The type 1 copper ions are thought to bind to the carboxy-terminal portion of the domains of ceruloplasmin (domain residues 240-350; Fig. 4) based on sequence homology with the type 1 copper-binding protein plastocyanin[27]. The four amino acid side chains proposed as the ligands for the type 1

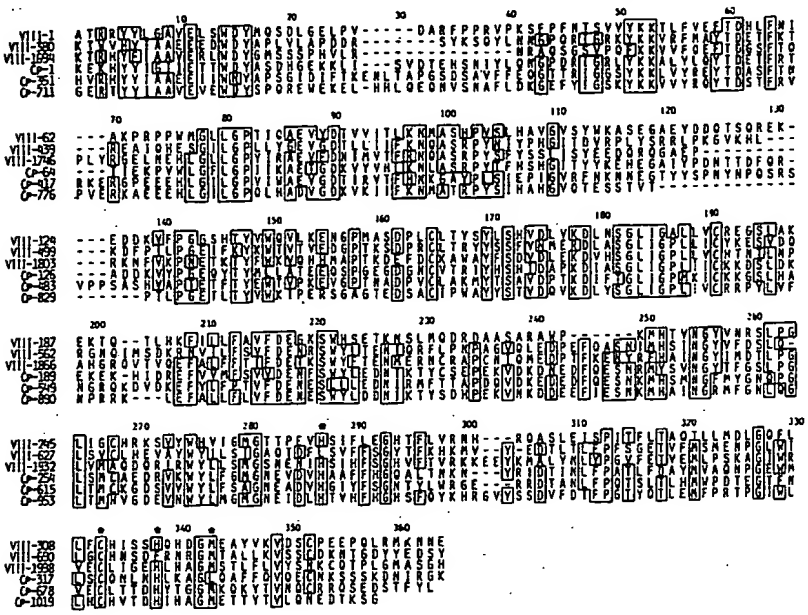

Fig. 4 Domain homology of human factor VIII and human ceruloplasmin[22]. Identical residues in four of the six domains are boxed. The protein is indicated to the left of the lines (VIII, factor VIII; Cp, ceruloplasmin) with the associated number indicating the position in the sequence of the first amino acid of the line. A domain residue numbering system is shown above the sequences. * Residues believed to be the ligands for type 1 copper (based on homology to plastocyanin[27]).

copper atoms of ceruloplasmin are found in the first and third A domains of factor VIII (histidines 287 and 338, cysteine 333 and methionine 343; see Fig. 4). The conservation of the copper-ligand residues found in ceruloplasmin strongly suggests similar metal binding characteristics for factor VIII.

Thrombin cleavage

The coagulant activity of factor VIII is increased markedly by treatment with catalytic amounts of the serine protease thrombin[28]. Thrombin activation of factor VIII is associated with a series of polypeptide cleavages[13-17]. Further incubation with thrombin leads to degradation of the protein with a concomitant loss of coagulant activity[13-17]. To understand the structural basis for these observations, the separated pools of factor VIII fragments were subjected to thrombin digestion and the resulting products characterized by SDS-polyacrylamide gel electrophoresis. Thrombin digestion of the M_r 80,000 protein resulted in a product of M_r 73,000 (Fig. 5A). Treatment of the polypeptide pool of M_r 90,000–210,000 led initially to the appearance of two bands of M_r 43,000 and 50,000 (Fig. 5A). Longer incubation with thrombin resulted in the conversion of the M_r 50,000 fragment to polypeptides of M_r 30,000 and 20,000 (data not shown). The M_r values of these thrombin digest fragments are similar to those generated by thrombin treatment of native factor VIII preparations[13,15,16]. Amino-terminal sequence analysis was performed on the separated protein chains before and after thrombin digestion. The resulting sequences are compared with the corresponding amino acid sequences deduced from the factor VIII cDNA sequence[19] in Fig. 5B; also shown are the potential cleavage sites found at the amino-terminus of the M_r 80,000 protein and that which separates the M_r 90,000 fragment from the carboxy-terminal portion of the M_r 210,000 protein. The sequence surrounding the latter potential cleavage site (position 740) is similar to the amino-terminal sequence of the M_r 70,000 protein (-PRSF---- RH-) (Fig. 5B). There is no other consensus sequence that would predict the specificity of thrombin cleavage. A homologous stretch preceeding the thrombin cleavage sites for the M_r 43,000 and 73,000 proteins is observed (Fig 5B), but whether this homology determines thrombin specificity or simply reflects the internal duplication is uncertain. The most consistent sequence found at thrombin cleavage sites within factor VIII is an arginine residue followed by either serine or alanine. Other such sequences (-RS- or -RA-) do occur within the protein but are not cleaved, suggesting the possible involvement of secondary structure in thrombin specificity. The cleavage that frees the M_r 80,000 protein occurs at an arginine-glutamic acid sequence, probably not a thrombin-generated cleavage site. This cleavage occurs quickly and is complete within the time required for isolation of the protein. The precursor factor VIII protein therefore may be cleaved to free the M_r 80,000 polypeptide by a protease other than thrombin.

Discussion

The structure of factor VIII revealed by the amino acid sequence predicted from the cloned cDNA and the structural characterization of polypeptide fragments described here are summarized in Fig. 6. The size of the factor VIII precursor moiety is consistent with the reported isolation of single-chain M_r 330,000 protein from plasma[18] and supports the notion that the protein circulates as a high-molecular weight form that is readily cleaved in plasma and/or during isolation to a series of degradation products.

The primary structure of factor VIII exhibits three distinct types of structural domain, including a triplicated region of ~330 amino acids (A domains), a unique region of 980 amino acids (the B domain) and a carboxy-terminal duplicated region of 150 amino acids (C domains), which are arranged in the order A1-A2-B-A3-C1-C2 (Fig. 6). The A domains of factor VIII show significant homology to ceruloplasmin, consisting also of a triplicated structure of three A domains but lacking both B and C domains[22-24]. Particularly striking is the clustering of cysteine residues at similar locations within related structural domains of factor VIII (Fig. 6). The determination of disulphide pairings for ceruloplasmin[23,29] predicts two types of internal disulphide bonding arrangements for the A domains of factor VIII. The disulphide structure proposed for the C domains of factor VIII is based on the proposition that the disulphide linkgages form between the two cysteine residues found in each domain. The large B domain which separates the second and third A domains of factor VIII contains only four cysteine residues, but the presence of 19 asparagine-linked glycosylation sites suggests that this region is extensively modified by carbohydrate addition.

The activation of factor X by factor IX_a in conjunction with factor VIII is known to require calcium ions. Factor IX_a and factor X both contain γ-carboxyglutamic acid residues which are thought to be involved in calcium binding. The protein-bound calcium ions mediate the interaction of these proteins with the phospholipid surface. The homology of factor VIII with ceruloplasmin suggests the possible involvement of copper or other metal ions in the role of factor VIII in factor X activation. One possibility for the role of such metal ion involvement is suggested by the binding of lanthanide ions by γ-carboxyglutamic acid residues[30]. It is interesting to speculate that the potential copper-binding ligands of factor VIII interact with a metal ion jointly bound by the γ-carboxyglutamate

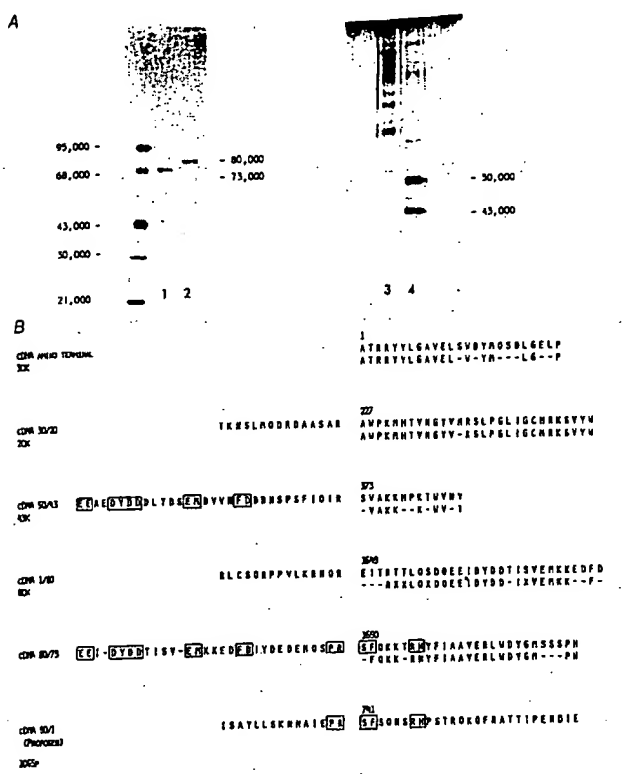

Fig. 5 *A*, Thrombin cleavage of separated factor VIII polypeptide fractions. *B*, Alignment of thrombin-generated amino termini with the deduced amino acid sequence[19].
Methods: *A*, The factor VIII polypeptides were separated as described in legend to Fig. 2*A*. SDS was removed by dialysis of the fractions against 8 M urea solutions and urea removed by dialysis against 0.01 M Tris, *p*H 8.0. Thrombin was then added at weight ratios to a maximum of 1:20. The digestions were allowed to proceed at room temperature and the extent of cleavage was monitored by SDS-gel electrophoresis. Lanes 1 and 2 are the M_r 80,000 protein with and without thrombin, respectively; lanes 3 and 4 are the M_r 90,000–210,000 protein without and with thrombin, respectively. *B*, Thrombin digestion products were separated by preparative SDS-gel electrophoresis, electroeluted by the procedure of Hunkapiller *et al.*[41] and analysed on a Beckman spinning cup sequencer[40]. The M_r 80,000 protein was obtained by TSK separation as described for Fig. 2*A*. The thrombin cleavage site is indicated by a space in the cDNA deduced sequence. The number above the subsequent amino acid corresponds to the position of that residue in the linear sequence. The amino-terminal protein sequences for the various polypeptide chains are aligned under the translated gene sequence. –, Positions where no residue could be identified; X, positions where the wrong amino acid was determined. The relative molecular masses of the proteins separated are listed (30K is the amino terminus obtained for the gel-eluted polypeptide with a M_r of 30,000); solidi indicate cleavage products (for example, 50/43 indicates the cleavage which separates the M_r 50,000 and 43,000 species). Regions which share sequence homology are boxed.

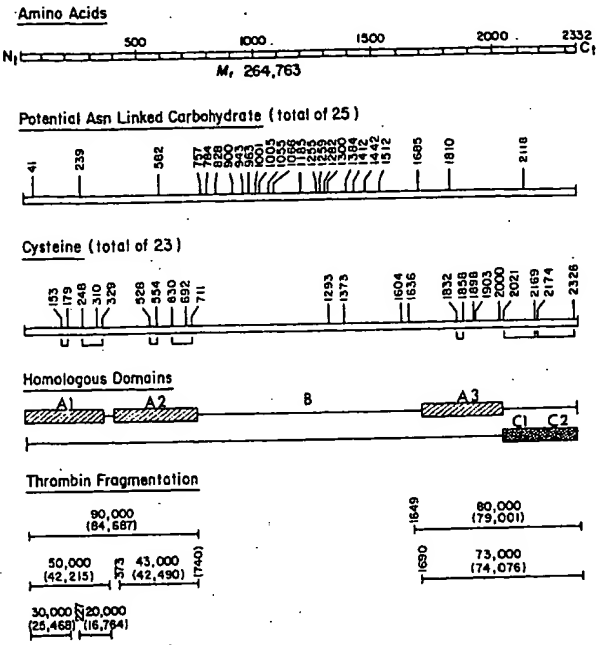

Fig. 6 Line diagram of factor VIII precursor protein. The molecular masses of the various fragments as determined by SDS-polyacrylamide gel electrophoresis are indicated (the M_r calculated from the actual amino acid composition[19] is given in parentheses). The positions of potential asparagine-linked glycosylation sites and cysteine residues are shown. The positions of the thrombin fragments and residue numbers of amino-terminal amino acids are also given. The position of cleavage of the M_r 90,000 protein has not been determined and is suggested. Boxes, the triplicated domains homologous to ceruloplasmin as well as the duplicated domain.

residues of factors IX or X. In addition to copper transport and haemostatis, several enzymatic functions have been ascribed to ceruloplasmin, including ferroxidase activity, amino oxidase activity and superoxide dismutase activity[31–33]. It will be important to determine whether any of these activities are associated with factor VIII.

The amino-terminal sequence of factor VIII thrombin fragments and the homology of factor VIII with ceruloplasmin provide insight into a functional purpose for the cleavages. Factor VIII isolated from plasma is usually degraded. In certain preparations, small amounts of a M_r 330,000 protein were observed when analysed on SDS-polyacrylamide gels run under non-reducing conditions; this protein was not observed when the factor VIII samples were reduced before electrophoresis. This reducible 330,000 may be a disulphide-linked, limited proteolytic degradation product of a single-chain M_r 330,000 factor VIII molecule first reported by Tuddenham *et al.*[18]. The portion of the protein immediately prior to the M_r 80,000 protein may thus be a disulphide linker region which holds the M_r 210,000 and 80,000 regions (or larger precursors) in the reducible 330,000 form. Exposure of plasma-derived protein preparations to thrombin results in activation of the factor VIII coagulant activity and the appearance of the band at M_r 90,000 (residues 1–740) due to the removal of the B domain or its fragments. Thrombin further cleaves both the M_r 80,000 and 90,000 proteins. The M_r 80,000 protein is cleaved after residue 1,689, releasing a M_r 4,500 peptide containing one potential asparagine glycosylation site. This peptide is highly acidic, containing 15 aspartic and glutamic acid residues and only 4 lysine or arginine residues out of a total of 41 amino acids. Thrombin cleavage of the M_r 90,000 protein to M_r 50,000 and 43,000 products occurs between the first two A domains of factor VIII after an acidic spacer region (15 aspartic and glutamic acid residues; 4 lysine/arginine; total of ~42 amino acids) having some sequence homology with the acidic peptide cleaved from the M_r 80,000 protein (Fig. 5*B*). Further cleavage of the M_r 50,000 protein (domain residue 240, Fig. 4) occurs immediately preceding the region having sequence homology with plastocyanin[27] thereby freeing this potential metal-binding domain.

Factor VIII has many properties in common with coagulant protein factor V[34–36]. These proteins function in the intrinsic coagulation cascade with an activated vitamin K-dependent coagulation protein (factors IX_a and X_a for factors VIII and V, respectively), a phospholipid surface and calcium ions. These complexes result in the specific activation of a second vitamin K-dependent coagulation protein (factor X and prothrombin for factors VIII and V, respectively). Factor VIII and factor V are both proteins of $M_r > 300{,}000$; they are cleaved by a series of thrombin-catalysed events to generate proteins of M_r 90,000 (from the amino termini) and M_r 80,000 (from the carboxy

termini) from a single-chain circulating form. By analogy with factor V, the M_r 90,000 and 80,000 proteins would correspond to fragments D and E of factor V, respectively[34]. These two fragments of factor V can be separated from the activation peptides and isolated as a functional two-subunit protein[35,36]. Both subunits are required for factor V activity[35] and both may be required for factor VIII activity. A highly glycosylated intermediate region is cleaved from both proteins. Therefore, both factors V and VIII seem to be highly similar in structure, thrombin cleavage pattern and, presumably, function.

The studies described here provide a structural basis for defining the role of the diverse molecular forms of factor VIII in their interaction with other proteins of the coagulation cascade. The availability of complete factor VIII cDNA clones capable of programming recombinant factor VIII synthesis in mammalian cell cultures[19] will offer a unique opportunity to perform similar studies with the single-chain precursor molecule. The questions raised here concerning the relationship of processing events, structural domains and homology to ceruloplasmin with the biological function of factor VIII may be answered by studying structural changes introduced into the protein by modification of these cloned DNA sequences.

We acknowledge the support of Speywood Laboratories Ltd. We thank Dr John Bell, Alice Kleiss and William Henzel for laboratory assistance; Gary Hooper for his assistance in the coordination of the project between various groups and companies; and Robert A. Swanson and Dr David W. Martin Jr for their encouragement.

Received 13 August; accepted 27 September 1984.

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Molecular cloning of a cDNA encoding human antihaemophilic factor

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A complete copy of the mRNA sequences encoding human coagulation factor VIII:C has been cloned and expressed. The DNA sequence predicts a single chain precursor of 2,351 amino acids with a relative molecular mass (M,) *267,039. The protein has an obvious domain structure, contains sequence repeats and is structurally related to factor V and ceruloplasmin.*

HAEMOPHILIA A is a bleeding disorder caused by deficiency or abnormality of a particular clotting protein, factor VIII:C[1] occurring in about 10–20 males in every 100,000. Afflicted individuals suffer episodes of uncontrolled bleeding and are treated currently with concentrates rich in factor VIII:C derived from human plasma. The available therapy, although reasonably effective, is very costly and is associated with a finite risk of infections. We report here significant progress in the use of recombinant DNA technology to provide pure human factor VIII:C as an alternative treatment for haemophiliacs.

Blood clotting begins with injury to a blood vessel. The damaged vessel wall causes adherence and accumulation of platelets activating the plasma proteins which initiate the coagulation process. Sequential activation, via specific proteolytic cleavages and conformational changes, of a series of proteins comprising the coagulation cascade eventually leads to deposition of insoluble fibrin which, together with aggregated platelets, curtails the escape of blood through the damaged vessel wall. Factor VIII:C is a large plasma glycoprotein that functions in the blood coagulation cascade as the cofactor for the factor IXa-dependent activation of factor X. It can be activated proteolytically by a variety of coagulation enzymes including thrombin[2].

In order to provide factor VIII:C for treatment of haemophiliacs we cloned a full-length cDNA. A major obstacle to the cloning effort was the large size of the protein, estimated to be at least M_r 250,000. Purification of factor VIII:C from plasma[3] is made difficult by its low abundance, its extreme sensitivity to degradation by serum proteases and its tight association with polymeric forms of the more abundant protein, von Willebrand factor. Fass *et al.*[4] have described a purification procedure for porcine factor VIII:C using monoclonal antibody